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Leukotriene E₄ is a full functional agonist for human cysteinyl leukotriene type 1 receptor-dependent gene expression

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Leukotriene E₄ (LTE₄) the most stable of the cysteinyl leukotrienes (cysLTs) binds poorly to classical type 1 (CysLT₁) and 2 (CysLT₂) receptors although it induces potent responses in human airways *in vivo*, such as bronchoconstriction, airway hyperresponsiveness and inflammatory cell influx suggesting the presence of a novel receptor that preferentially responds to LTE₄. To identify such a receptor two human mast cell lines, LAD2 and LUVA, were selected that differentially responded to LTE₄ when analysed by intracellular signalling and gene expression. Comparative transcriptome analysis and recombinant gene overexpression experiments revealed CysLT₁ as a receptor responsible for potent LTE₄-induced response in LAD2 but not in LUVA cells, an observation confirmed further by gene knockdown and selective inhibitors. Lentiviral overexpression of CysLT₁ in LUVA cells augmented intracellular calcium signalling induced by LTE₄ but did not restore full agonist responses at the gene expression level. Our data support a model where both an increased expression of Gα_q-coupled CysLT₁, and sustained intracellular calcium mobilisation and extracellular signal-regulated kinase (Erk) activation, are required for LTE₄-mediated regulation of gene expression in human cells. Our study shows for the first time that CysLT₁ expression is critically important for responsiveness to LTE₄ within a human cell system.

Cysteinyl leukotrienes (cysLTs) (LTC₄, LTD₄ and LTE₄) play pivotal roles in cell proliferation, differentiation, migration and regulation of immune responses implicated in a wide variety of disorders, including asthma, allergy, atherosclerosis and cancer¹. CysLTs are products of the 5-lipoxygenase (5-LO) pathway. 5-LO converts arachidonic acid to an unstable intermediate LTA₄, which is then conjugated to reduced glutathione by leukotriene C₄ synthase to form LTC₄. After transport to the extracellular space LTC₄ is converted to LTD₄ and then to the terminal product LTE₄, the most abundant cysLT in biological fluids. The biological actions of cysLTs are mediated by 2 currently identified G-protein coupled receptors (GPCR): cysLT type 1 (CysLT₁) and 2 (CysLT₂) receptors. They differ in binding affinities for different cysLTs. CysLT₁ is recognized as a high-affinity receptor for LTD₄, whereas CysLT₂ binds LTC₄ and LTD₄ with similar affinity. LTE₄, the most stable of the cysLTs, binds poorly to the classical CysLT₁ and CysLT₂ and is also much less potent than LTC₄ and LTD₄ in inducing cellular responses *in vitro*, showing a partial agonistic activity^{2–5}. However *in vivo*, it is LTE₄ that has shown to be the most potent cysLT in eliciting influx of eosinophils and basophils into bronchial mucosa of asthmatic subjects and in enhancing airway responsiveness to histamine and increasing vascular permeability, suggesting the existence of one or more leukotriene receptors that have not been identified to date^{6–10}. The potential presence of such a receptor has been demonstrated in CysLT₁/CysLT₂ double knock-out mice¹¹ but human data are lacking.

The observations that asthmatic airways respond with enhanced bronchoconstriction to inhaled cysLTs, especially to LTE₄ in comparison with normal subjects¹⁰ and that infiltration of airways by mast cells is associated with disordered airway function in asthma¹² suggest that mast cells could be a potential target cell type expressing a putative receptor preferentially responding to LTE₄. In fact, the possible existence of such a novel,

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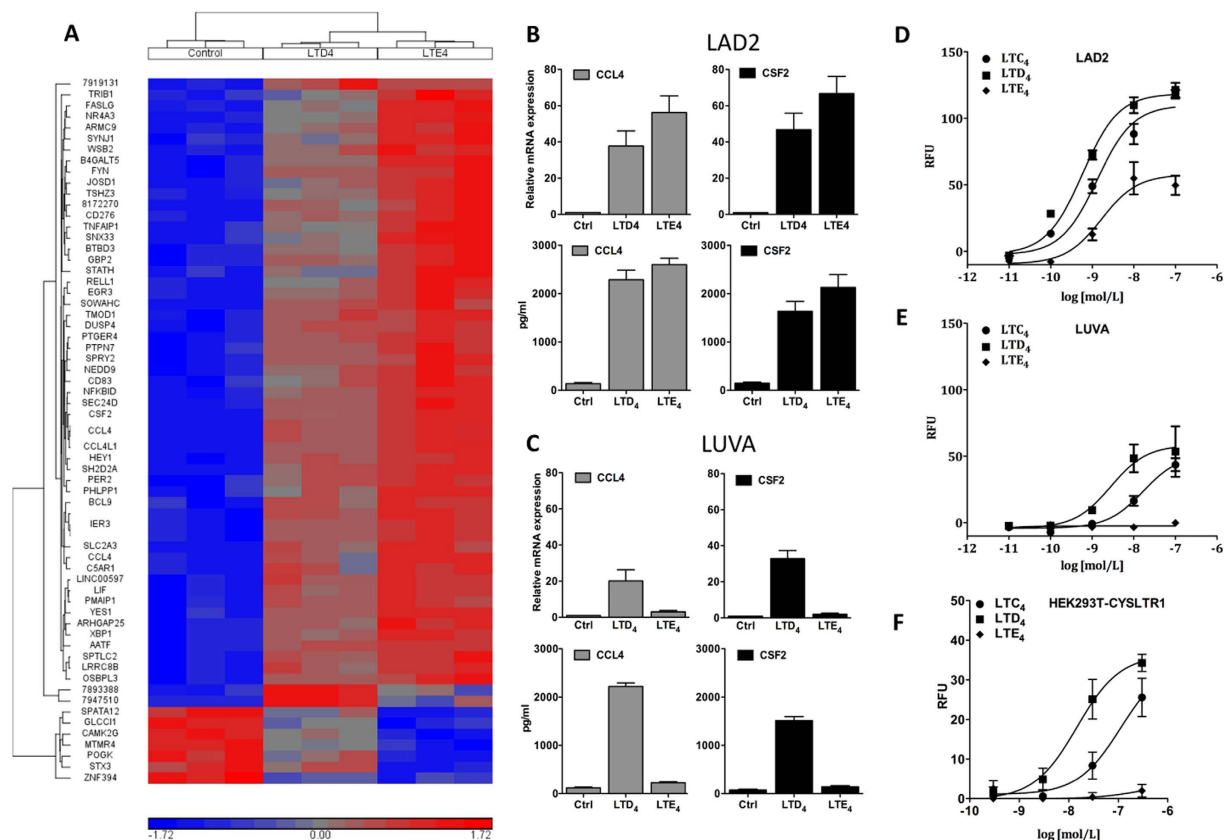


Figure 1. LTE₄ is a potent agonist in LAD2 but not in LUVA cells. (A) LAD2 cells (n = 3) were stimulated with vehicle control, LTD₄ and LTE₄ and gene expression was analysed using microarrays. Hierarchical clustering of significantly (ANOVA, $p < 0.05$ LTD₄ and LTE₄ compared to control, False Discovery Rate = 0.1) regulated genes is presented as a heat map. (B) LAD2 and (C) LUVA cells were stimulated and CCL4 or CSF2 gene expression were measured at mRNA and protein levels. Data expressed as mean \pm SEM from 3 separate experiments. (D) LAD2, (E) LUVA and (F) HEK293T transfected with CYSLTR1 cells were stimulated with indicated concentrations of LTC₄, LTD₄ and LTE₄ and calcium mobilisation was measured. Data from 3 experiments run in triplicate, presented as mean \pm SEM of baseline corrected peak intracellular calcium response. Relative fluorescence unit (RFU).

LTE₄-activated receptor has been suggested in human mast cells¹³. In this study LTE₄ has been shown to be the most potent of cysLTs in inducing cell proliferation and activation of gene expression in human primary mast cells and LAD2 human mast cell line. LTE₄-mediated activities were resistant to knockdown of CysLT₁ and CysLT₂ but were dependent on PPAR- γ signalling. Another study has suggested that the P2Y12 receptor is required for LTE₄-mediated responses¹⁴ but these observations have not been confirmed¹⁵.

In order to identify such a receptor responding to LTE₄ we studied human mast cells and used transcriptome profiling by microarrays, recombinant GPCR overexpression models and methods analysing GPCR signalling. We characterize LTE₄ as a fully functional agonist activating human CysLT₁ and show for the first time that CysLT₁ expression is critically important for responsiveness to LTE₄ within a human cell system.

Results

LTE₄ signals differently in LAD2 and LUVA cells. LTE₄ has been shown to induce potent responses in LAD2 cells¹³ offering a model for identification of the elusive receptor responsible for LTE₄ signalling. In order to compare responses between LTD₄ and LTE₄ microarray analysis of LAD2 cells stimulated with either vehicle control, LTD₄ or LTE₄ was carried out in the presence of L-cysteine (3 mmol/L) to inhibit dipeptidase enzyme responsible for converting LTD₄ to LTE₄¹⁶. Both leukotrienes significantly regulated expression of 64 genes including many chemokines, growth and transcription factors (Fig. 1A and supplementary Table 1). LTE₄ was more potent in up and down regulation of gene expression than LTD₄ for the majority of analysed genes, providing strong evidence for a robust LTE₄ response in LAD2 cells. CCL4 and CSF2 were among the most upregulated genes in LAD2 cells and were selected for further analysis. qRT-PCR and ELISA analysis of LAD2 cells showed induction of CCL4 and CSF2 with LTE₄ consistently matching or being the more potent of the 2 ligands (Fig. 1B). To verify whether this responsiveness to LTE₄ is characteristic for other mast cells, another human mast cell line, LUVA, was analysed to compare responses to LTD₄ and LTE₄. Although in LUVA cells LTD₄ regulated gene expression in a similarly potent way to LAD2 cells, LTE₄ induced only very weak responses (Fig. 1C). As intracellular calcium mobilisation is a secondary messenger signalling cue for classical leukotriene receptors, cysLT induced

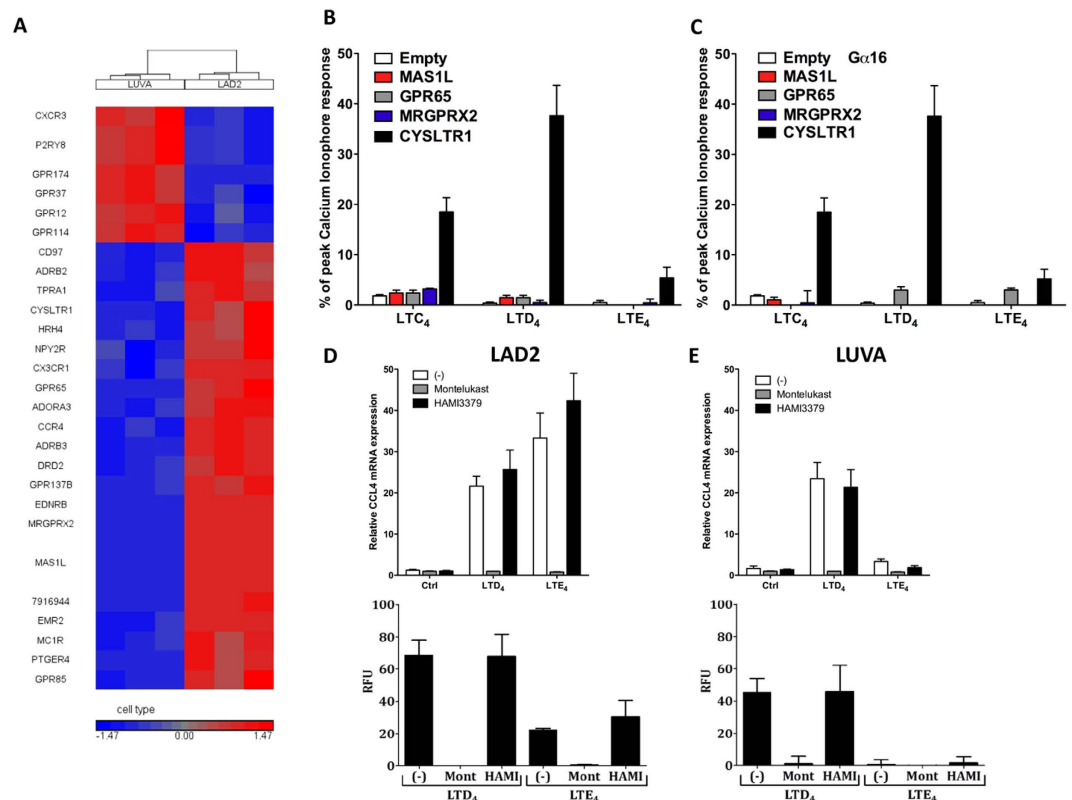


Figure 2. Comparison of GPCR gene expression profiles between LAD2 and LUVA cells. (A) Microarray gene expression was compared in LAD2 and LUVA cells ($n = 3$) and hierarchical clustering of differentially expressed GPCRs (ANOVA, $p < 0.05$, > 2 fold difference) is presented as a heat map. Intracellular calcium mobilisation was analysed in HEK293T cells transiently transfected with the genes of interest (B) and co-transfected with $G\alpha_{16}$ (C). Data expressed as percentage of peak calcium ionophore response, mean \pm SEM from 3 experiments run in triplicate. LAD2 (D) and LUVA (E) cells were pre-treated with Montelukast (100 nmol/L) and HAMI3379 (1 μ mol/L) for 10 minutes, stimulated with LTD₄ and LTE₄ (both 100 nmol/L) and CCL4 mRNA expression or calcium mobilisation was measured. Data expressed as a fold difference in comparison to vehicle control or as baseline corrected peak calcium response. Mean \pm SEM from 3 separate experiments. Relative fluorescence unit (RFU).

calcium mobilisation was analysed in both cell lines. In LAD2 cells, all cysLTs induced a concentration-dependent calcium mobilisation (Fig. 1D), with LTD₄ and LTC₄ showing similar potency (LTC₄ EC₅₀ 1.3×10^{-9} M, LTD₄ EC₅₀ 0.58×10^{-9} M) and LTE₄ being the weakest of all 3 ligands but still inducing a robust response (LTE₄ EC₅₀ 1.67×10^{-9} M). In contrast, LTD₄ was the most potent ligand in LUVA cells (EC₅₀ 2.8×10^{-9} M) followed by LTC₄ (EC₅₀ 1.7×10^{-8} M), while LTE₄ induced very weak response (EC₅₀ not determined) (Fig. 1E). Similar potencies of cysLTs as in LUVA cells were detected in HEK293T cells transfected with human CYSLTR1 (Fig. 1F) (LTC₄ EC₅₀ 1.12×10^{-8} M, LTD₄ EC₅₀ 0.9×10^{-9} M; LTE₄ EC₅₀ 8.32×10^{-8} M). Therefore LAD2 and LUVA cells represent two human mast cell lines that respond differently to LTE₄ stimulation.

Comparison of GPCR gene expression profiles between LAD2 and LUVA cells. A previous study¹³ has suggested that in LAD2 cells LTE₄ signals through a novel, CysLT₃ receptor, different from classical CysLT₁ and CysLT₂. As our observations in LAD2 and LUVA cells indicated that a potential LTE₄ receptor should be differentially expressed in LAD2 and LUVA cells, gene expression was compared between LAD2 and LUVA cells using microarray in order to identify the putative gene. A list of significantly differentially expressed genes (ANOVA $p < 0.05$; > 2 fold difference) was generated and GPCR genes were filtered using the IUPHAR GPCR database¹⁷. Among 27 GPCRs that differed significantly in expression between LAD2 and LUVA cells (Fig. 2A, supplementary Table 2), 10 GPCRs were considered orphan receptors (without known ligands) (GPR12, GPR37, GPR65, GPR85, GPR114, GPR137B, GPR174, MAS1L, MRGPRX2 and P2RY8). GPR65, MAS1L and MRGPRX2 were the most differentially expressed orphan GPCRs (9.9, 32.4 and 70.2 fold difference between LAD2 and LUVA cells, respectively). To ascertain whether cysLTs, and LTE₄ in particular, could mediate signalling through any of these receptors, plasmids encoding GPR65, MAS1L and MRGPRX2 were transiently transfected into HEK293T cells and calcium mobilisation was analysed upon stimulation with cysLTs (Fig. 2B). CYSLTR1 gene was among differentially expressed GPCRs and was used as a positive control for all experiments. No specific calcium responses were observed in any of the transfectants apart from cells transfected with CYSLTR1, which showed the predicted pattern of response to cysLTs. As co-transfections of GPCRs and $G\alpha_{16}$ have been

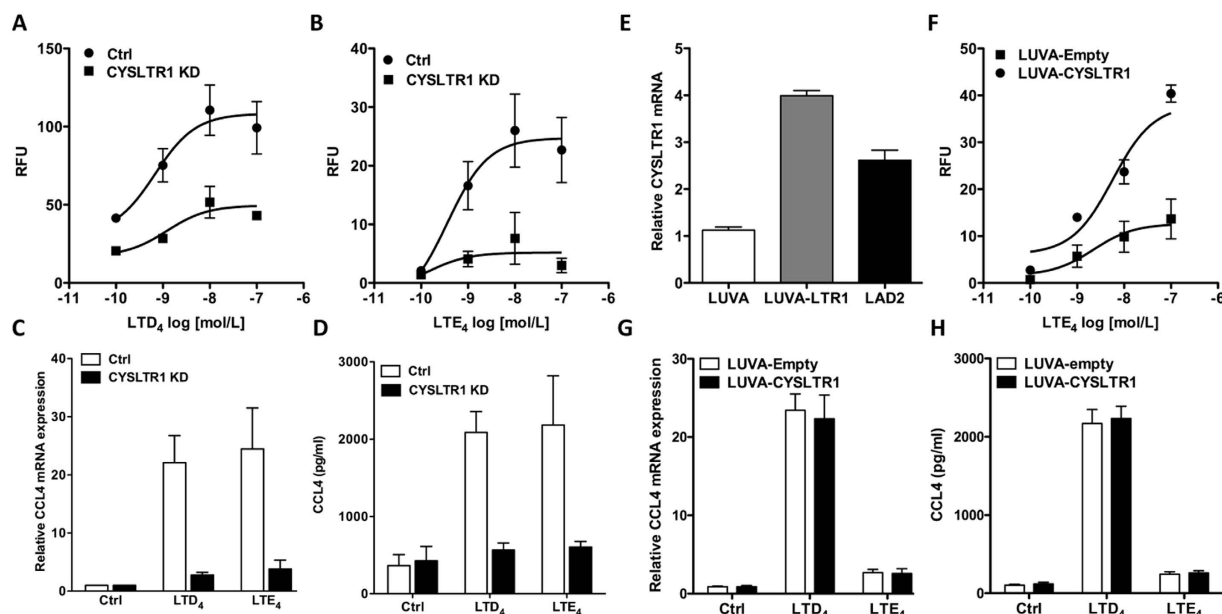


Figure 3. CysLT₁ is required for LTE₄ induced signalling in LAD2 cells. Calcium mobilisation responses to LTD₄ (A) and LTE₄ (B) in Empty control (Ctrl) and CYSLTR1 knocked down (CYSLTR1 KD) LAD2 cells. Baseline corrected peak calcium responses from 3 experiments run in triplicate presented as mean \pm SEM. (C) Control and CYSLTR1 knocked down LAD2 cells were stimulated with vehicle control, LTD₄ or LTE₄ for 2 (mRNA) (C) or 6 hours (protein) (D) before analysis. Data expressed as fold difference in comparison to vehicle control for CCL4 mRNA and as CCL4 supernatant concentrations. Mean \pm SEM from 3–5 experiments, relative fluorescence unit (RFU). (E) LUVA cells were stably transduced with empty (LUVA-empty) or CYSLTR1 overexpression (LUVA-CYSLTR1) vectors and relative CYSLTR1 mRNA expression was measured and compared to LAD2 cells. Mean \pm SEM, n = 6. (F) Calcium mobilisation response to a range of LTE₄ concentrations was evaluated in empty control and CYSLTR1 transduced LUVA cells. Mean \pm SEM of baseline corrected peak calcium responses, n = 9. Control empty vector and CYSLTR1 transduced LUVA cells were stimulated as indicated before CCL4 mRNA (G) or protein (H) expression was measured. Mean \pm SEM of 3 separate experiments.

reported previously to direct signal transduction to phospholipase C and calcium signalling¹⁸, target genes were co-expressed with human G α_{16} and responses to cysLTs measured using calcium mobilisation in order to analyse potential alternative GPCR signalling pathway. Similarly, no response was observed in any of our overexpression models apart from CYSLTR1 transfected cells (Fig. 2C). Thus CysLT₁ was the receptor that was differentially expressed in LAD2 and LUVA cells (4.3 fold difference) and responded to cysLTs.

CysLT₁ is required for LTE₄ induced signalling in LAD2 cells. To determine whether CysLT₁ could be involved in LTE₄ signal transduction, LAD2 and LUVA cells were pretreated with selective CysLT₁ and CysLT₂ antagonists, Montelukast and HAMI3379, respectively. Antagonists' selectivity was previously verified in HEK293T cell transfection models (supplementary Figure 1). qRT-PCR analysis of CCL4 gene expression in LAD2 cells showed that both LTD₄ and LTE₄ induced responses were fully inhibited by Montelukast while HAMI3379 had no effect (Fig. 2D). In LUVA cells, LTD₄ signalling was again fully inhibited by Montelukast but not by HAMI3379 (Fig. 2E). Analysis of calcium mobilisation in these cells showed a very similar picture, with Montelukast fully inhibiting LTE₄ responses in LAD2 as well as LTD₄ responses in LAD2 and LUVA cells while HAMI3379 had no effect (Fig. 2D,E). To verify whether the potent LTE₄ induced, Montelukast sensitive, response in LAD2 cells was attributable specifically to CysLT₁ signalling and not via another Montelukast sensitive receptor, stable CYSLTR1 receptor knockdown was generated in LAD2 cells using shRNA. Four shRNA targeting different regions of CYSLTR1 were transduced into separate LAD2 cell populations using lentiviral particles. qRT-PCR analysis of CYSLTR1 revealed shRNA “475” to significantly knock down CYSLTR1, without affecting CYSLTR2 mRNA expression (supplementary Figure 2). Knocking down of CYSLTR1 substantially inhibited intracellular calcium responses to LTD₄ and LTE₄ (Fig. 3A,B), confirming a functional decrease in CysLT₁ expression. CCL4 and CSF2 mRNA and protein expression upon LTD₄ and LTE₄ stimulation were almost completely abrogated in CysLT₁ knocked down LAD2 cells (Fig. 3C,D and supplementary Figure 3) identifying CysLT₁ as a receptor responsible for LTE₄ induced signalling in LAD2 cells.

Overexpression of CysLT₁ in LUVA cells does not determine LTE₄ responses. Our GPCR expression profiles identified CYSLTR1 as more highly expressed in LAD2 than in LUVA cells. To test the hypothesis that the expression level of CYSLTR1 is relevant for mast cell responsiveness to LTE₄, CYSLTR1 was stably overexpressed in LUVA cells using lentiviral transduction and positive clones were selected using puromycin.

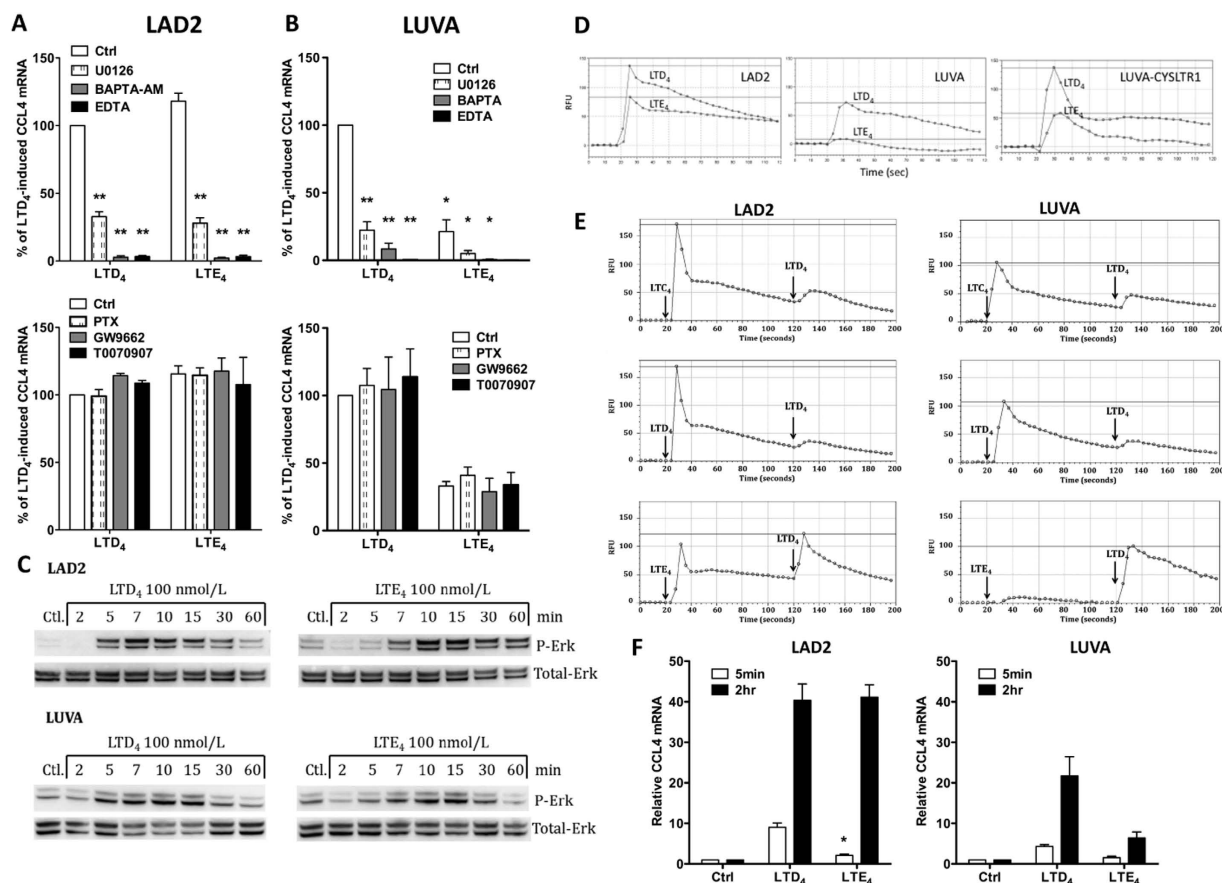


Figure 4. LTE_4 induces sustained signalling in LAD2 cells. LAD2 (A) and LUVA (B) cells were pre-treated with selected inhibitors and stimulated with LTD_4 or LTE_4 . Data from 3 separate experiments shown as % of LTD_4 -induced CCL4 mRNA expression (mean \pm SEM), * $p < 0.05$, ** $p < 0.001$, ANOVA with Bonferroni post test compared to LTD_4 or LTE_4 . (C) LAD2 and LUVA cells were stimulated for time indicated with vehicle control (Ctrl), LTD_4 or LTE_4 (both 100 nmol/L) and phosphorylated Erk and total Erk expression measured using specific antibodies. Results from a representative experiment of 3 performed. Calcium mobilisation traces of LAD2, LUVA or LUVA-CYSLTR1 cells stimulated as indicated with LTD_4 or LTE_4 (100 nmol/L) once (D) or twice (E). Representative of 3 separate experiments, relative fluorescence unit (RFU). Black arrows indicate start of stimulation. (F) LAD2 and LUVA cells were exposed for either 5 minutes or 2 hours to vehicle control, LTD_4 or LTE_4 (both 100 nmol/L) and CCL4 mRNA measured by qRT-PCR after 2 hours incubation. Mean \pm SEM data from 3 experiments shown as a fold change in comparison to controls. * $p < 0.05$, 2-way ANOVA comparison between 5 min LTD_4 and LTE_4 stimulations.

qRT-PCR confirmed a 3-fold increase in CYSLTR1 expression in the transduced population, a level similar to LAD2 cells (Fig. 3E). Functional CYSLTR1 overexpression was confirmed using calcium assay and showed potent concentration-dependent increase in LTE_4 induced calcium responses (Fig. 3F), again similar to responses observed in LAD2 cells. Stimulation of LUVA cells overexpressing CysLT₁ and control empty vector-transduced cells with either LTD_4 or LTE_4 revealed no significant differences in CCL4 mRNA or protein induction between both cell lines (Fig. 3G,H), showing that the expression level of CysLT₁ does not solely determine LTE_4 induced gene regulation, even though it allows for enhanced calcium mobilisation in response to LTE_4 .

Comparison of CYSLTR1 gene sequence between LAD2 and LUVA cells. As genetic variations in the CYSLTR1 gene between LAD2 and LUVA cells could account for such differential responses to LTE_4 , promoter and coding regions of CYSLTR1 in both cell types were sequenced. DNA was extracted and CYSLTR1 promoter fragment containing 4 single nucleotide polymorphisms (SNPs) (rs321029, rs2637204, rs2806489, rs7066737) as well as the entire coding region were PCR amplified and sequenced. BLAST analysis of DNA sequences from LAD2 and LUVA cells revealed no differences between cell lines and showed that both cell lines share the same promoter homozygous haplotype, “CAAC” for 4 SNPs studied, respectively and homozygous T allele for rs320995 coding synonymous SNP. Human CYSLTR1 gene is localized to chromosome X thus lack of heterozygosity at the locus was consistent with the fact that both cell lines were derived from male donors.

CysLT-activated CysLT₁ signals through G α_q , calcium and Erk for gene regulation. In order to compare CysLT₁ mediated signalling in LAD2 and LUVA, both cell types were pre-incubated with several signalling pathway inhibitors and gene expression was measured in response to LTD_4 and LTE_4 (Fig. 4A,B). LTD_4 - and

LTE₄-induced CCL4 mRNA expression was potently inhibited by U0126 (MEK/Erk pathway inhibitor), intracellular (BAPTA-AM) and extracellular (EDTA) calcium chelators but was not modified by pertussis toxin or GW9662 and T0070907 (PPAR- γ inhibitors), suggesting that in both cell lines CysLT₁ couples to G α q, requires intracellular and extracellular calcium and Erk activation for regulation of gene expression.

LTE₄ activates prolonged signalling in LAD2 cells. To further analyse the agonistic activity of LTE₄, time course experiments of Erk phosphorylation were conducted and analysed by Western blotting (Fig. 4C). In LAD2 cells stimulated with LTD₄, Erk phosphorylation peaked at 7 minutes with a gradual decrease until 60 minutes. LTE₄ induced a peak of Erk phosphorylation later but with a more sustained phosphorylation, still being detectable after 60 minutes. In LUVA cells the time point of highest Erk phosphorylation was similar to LAD2 cells but LTE₄-induced Erk phosphorylation was shorter than in LAD2 cells. Thus sustained Erk phosphorylation induced by LTE₄ in LAD2 but not in LUVA cells underlies an important difference in CysLT₁-mediated responses between the cell lines.

We next compared calcium mobilisation kinetics in LAD2 cells; although LTD₄ induced a higher peak response than LTE₄, the intracellular calcium levels decreased at a higher rate after LTD₄ stimulation while LTE₄ induced a long lasting plateau phase (Fig. 4D). The sustained calcium signalling in response to LTE₄ was not observed in LUVA cells or in LUVA cells overexpressing CysLT₁ (Fig. 4D).

As GPCR signalling is regulated through receptor desensitization, cross desensitization experiments with cysLTs were performed (Fig. 4E). Prior stimulation with either LTC₄ or LTD₄ completely abrogated calcium response to LTD₄ in LAD2 and LUVA cells, suggesting that both LTC₄ and LTD₄ can fully desensitize CysLT₁ in both cell lines. However, prior stimulation with LTE₄ caused only partial inhibition of the calcium response to LTD₄ in LAD2 and LUVA cells, showing partial agonistic/desensitizing activity of LTE₄ but no difference between the cell lines in LTE₄ mediated signalling. The sustained increased level of calcium in LAD2 but not in LUVA cells after LTE₄ stimulation was again the main difference observed between the cell lines in these experiments.

To analyse whether prolonged calcium/Erk signalling induced by LTE₄ in LAD2 cells affects gene expression, CCL4 mRNA expression was analysed in LAD2 and LUVA cells after short (5 minutes) and long (2 hours) term exposure to LTD₄ and LTE₄, respectively. In LAD2 cells, 2 hour exposure to LTE₄ and LTD₄, caused similar upregulation of CCL4 mRNA expression (Fig. 4F). Stimulation of LAD2 cells with LTE₄ for only 5 minutes failed to induce potent CCL4 expression with mRNA levels being significantly lower than that induced by LTD₄. In LUVA cells, no difference could be observed between different exposure times.

Discussion

This study identifies LTE₄ as a fully functional agonist activating human CysLT₁ for regulation of gene expression in LAD2 cells although only weak, partial agonism of LTE₄ signalling could be detected in LUVA cells. Our data suggest that increased expression of CysLT₁ and induction of prolonged intracellular signalling are required for LTE₄ functional agonism. Ever since the elucidation and cloning of human CysLT₁ and CysLT₂, LTE₄ has been considered as a final, non-active leukotriene metabolite due to its weak efficacy in recombinant systems and poor binding affinities compared to LTC₄ and LTD₄⁶. However, it was LTE₄ that was shown to be the most potent cysLT in inducing inflammatory and contractile responses in asthmatic subjects. Our observation that LTE₄ can induce full agonistic activity through CysLT₁ could be of relevance for explaining this discrepancy between potent *in vivo* activity of LTE₄ observed in asthmatic patients and weak *in vitro* potency for classical cysLT receptors. Early studies analysing the effects of cysLTs *in vivo* revealed a disproportionate augmentation in relative responses to LTE₄ inhalation in asthmatic patients when compared to healthy individuals¹⁰. LTE₄ responsiveness was increased more than 200 fold in asthmatics while responses to LTC₄ and LTD₄ were increased 6 and 9 fold respectively. More recent clinical studies suggest that CysLT₁ is more highly expressed in asthmatic airways compared to healthy individuals¹⁹, with further increase observed in asthma exacerbations and in a sub-phenotype of asthma, patients with aspirin-exacerbated respiratory disease (AERD)^{20,21}. This increased CysLT₁ expression observed in AERD patients was significantly decreased following successful aspirin desensitisation, a procedure associated also with a significant reduction in sensitivity to inhaled LTE₄²⁰. We found similar disproportionate augmentation in LTE₄-induced responses when comparing LAD2 and LUVA cells, a model of relatively high versus low CysLT₁ expression. LTC₄ and LTD₄ were 2–3 times more potent at inducing calcium mobilisation in LAD2 cells compared to LUVA while LTE₄ showed nearly 60-fold difference. Such potent responses to cysLTs, including LTE₄ have been recently described in other human primary cells expressing high levels of CysLT₁, T helper type 2 (Th2) lymphocytes^{22,23} and group 2 innate lymphoid cells (ILC2)²⁴ supporting further our observation.

Prolonged intracellular signalling was identified as another potential contributing factor for the potency of LTE₄ responses. The sustained increase in intracellular calcium and Erk phosphorylation upon LTE₄ stimulation were observed in LAD2 but not in LUVA cells, suggesting that prolonged signalling could be critical for transcriptional regulation. LTE₄, in contrast to LTC₄ and LTD₄, shows only partial activity and does not desensitize CysLT₁ responses, a feature that can contribute to prolonged signalling in response to LTE₄ in LAD2 cells. In fact, in experiments with short term exposure to agonists LTE₄ showed only weak, partial agonist activity in comparison to LTD₄, confirming important role of prolonged signalling in LTE₄ induced responses. Overexpression of CysLT₁ in LUVA did not restore sustained intracellular calcium and full agonism even though it increased peak calcium response to LTE₄, suggesting that additional unidentified signalling molecules expressed in LAD2 cells but not in LUVA, are also required for full functional agonism of LTE₄.

Mouse models provide strong evidence that CysLT₁ and CysLT₂ are not the only cysLT receptors as germline deletion did not diminish leukotriene-mediated inflammation^{11,14,25}. Our data presented here provide an explanation for potent LTE₄ activity observed in humans but do not rule out the possibility of another cysLT receptor. Our study shows for the first time that CysLT₁ expression is critically important for responsiveness to LTE₄ within

a human cell system. This could potentially be relevant for human cell types other than mast cells and could thus have important implications for diagnostics and targeted treatment of specific phenotypes of asthma.

Materials and Methods

Reagents. Leukotrienes (LTC₄, LTD₄ and LTE₄), Montelukast, MK-571, HAMI3379, U-0126, GW9662, T0070907 (all Cayman Chemical), EDTA (Ambion), BAPTA-AM, Pertussis Toxin, Calcium ionophore (A23187), (all Sigma-Aldrich) were obtained from the manufacturers.

Cell Culture. HEK293T cells were cultured in DMEM medium supplemented with 2 mmol/L glutamine, 10% fetal bovine serum and Penicillin/Streptomycin (50 units/ml) (all Life Technologies) in a humidified 5% CO₂ 37 °C incubator. LAD2 cells (a kind gift from Dr. Arnold Kirshenbaum, NIAID, NIH, USA²⁶) and LUVA cells (a kind gift from Dr. John Steinke, University of Virginia, USA²⁷) were cultured in StemPro-34 medium supplemented with L-glutamine (2 mmol/L), Pen/Strep (50 IU/ml) and with or without stem cell factor (SCF) (100 ng/ml) (all Life Technologies), respectively. Cells were hemidepleted weekly with fresh medium.

Transient transfections. HEK293T cells cultured to above 60% confluence were transiently transfected as described previously^{4,15} with a mixture of Lipofectamine 2000 (Life Technologies) and the following plasmids as indicated: pcDNA3.1-empty, pcDNA3.1-human CYSLTR1, pcDNA3.1-human CYSLTR2, pcDNA3.1-human GPR65, pcDNA3.1-human GNA15 (G α_{16}) (all the Missouri S&T cDNA Resource Center, Rolla, Mo) and pCMV6-Kan/Neo-human MAS1L and human MRGPRX2 (Origene Technologies) in serum-free medium (Opti-MEM, Life Technologies) according to manufacturer's protocol. After incubation the transfection medium was removed and HEK293T cells were cultured for 36 hours before calcium mobilisation was assayed in response to stimulation with calcium ionophore (1 μ mol/L), LTC₄, LTD₄ and LTE₄ (all 100 nmol/L).

Short hairpin RNA (shRNA) knockdown. For stable gene silencing shRNA constructs targeting different regions of human CYSLTR1 (clone ID: V3LHS_305475, V3LHS_305478, V2LHS_90946 and V2LHS_90947) were purchased from ThermoScientific and used to generate lentiviral particles with the lentiviral packaging system (psPAX2, pMD2.G and PEG-itTM precipitation) (System Biosciences) according to manufacturer's protocol. LAD2 cells were transduced with viral particles for 24 hours and positive cells selected using Puromycin (2 μ g/ml) (Life Technologies). Efficiency of transduction was assessed by analysing GFP expression using flow cytometry.

CYSLTR1 overexpression. CYSLTR1 gene was amplified from the pcDNA3.1-CYSLTR1 construct (UMR cDNA Resource Center) with primers containing restriction enzyme sites for NheI and BamHI (5'-AGGTGCTAGCATGGATGAAACAGGAAATT and 5'-GCGGGGATCCCTATACCTTACATATTTTC) and cloned into lentiviral vector pCDH (System Biosciences) encoding GFP and puromycin resistance under the EF1 promoter and a multiple cloning site under the CMV promoter. Viral particles were generated using lentiviral packaging system (System Biosciences). LUVA cells were transduced, selected with puromycin (2 μ g/ml) and transduction efficiency was evaluated by GFP expression using flow cytometry.

DNA sequencing. Total DNA was extracted using DNeasy Tissue kit (Qiagen) and fragments of CYSLTR1 gene were amplified using Platinum Taq Polymerase High Fidelity (Invitrogen) following manufacturer's protocol and primers: CYSLTR1 promoter 5'-AACTGGAGACTTGCAGGTTGCG, 5'-AACATCAAAGTGCTGCCCCAGG; CYSLTR1 coding region 5'-TCAATGCCTCACTACTATTGCTTG, 5'-TTGGTTTGGACTGGAAATGGG and sequenced by Source Bioscience Sanger service using custom designed primers: CYSLTR1 promoter 5'-TAAGATGGGAAGCAGGGACG, 5'-GGCTTCAATCAGCACATACC; CYSLTR1 coding region 5'-ATACCAAGTGCTTTGAGCC, 5'-GCATTGGCTCTTTGGTG and 5'-GTTTGATTGTCTTGTGGGG.

Calcium mobilisation assay. Calcium mobilisation assays were conducted using FLIPR calcium 4 assay kit (Molecular Devices) as described previously^{4,28}. Cells (1.5 \times 10⁵/well) were plated into poly-L-lysine coated 96 well plates in RPMI 1640 supplemented with 10 mmol/L HEPES, incubated for 1 hour with FLIPR loading buffer prior to addition of ligand and fluorescent intensity was measured at 37 °C using a Flexstation 3 (Molecular Devices). Controls included medium control with ethanol for leukotriene stimulations. Results were analysed with SoftMax Pro Software (Molecular Devices).

Real time PCR. LAD2 and LUVA cells were stimulated for 2 hours in the presence of L-cysteine (3 mmol/L) with LTD₄ and LTE₄ (both 100 nmol/L) and vehicle control. In some experiments as indicated cells were pre-treated with U0126 (1 μ mol/L; 30 min), BAPTA-AM (30 μ mol/L; 30 min), EDTA (2.5 mmol/L; 5 min), pertussis toxin (PTX) (100 ng/ml; overnight), GW9662 (10 μ mol/L; 30 min) or T0070907 (1 μ mol/L; 30 min). Total cellular RNA was isolated using the miRNeasy mini kit (Qiagen), DNAase treated (Ambion) and reverse transcribed using RevertAid M-MuLV (Fermentas). Expression of mRNA encoding selected genes was measured using real time PCR on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Commercially available primer probe sets: 18S rRNA - 4319413E (Applied Biosystems) and individually designed assays using the Universal Probe Library (UPL) (Roche): CYSLTR1- probe 71, primers 5'-GGAGAGGGTCAAAGCAACAA, 5'-TGCAGAAGTCCGTGGTCATA; CYSLTR2- probe 21, primers 5'-TGATGTGACACTGCCGTTCT, 5'-TCATGGCTTCCTCAATAATGC; CCL4- probe 20, primers 5'-CAGCACAGACTTGCTTGCTT, 5'-CTTCCTCGCAACTTTGTGGT; CSF2- probe 1, primers 5'-GCCCTTGAGCTTGGTGAG, 5'-TCTCAGAAATGTTTGACCTCC were used. All primers/probes were tested for optimal efficiency of amplification. Relative gene expression was normalized to 18S rRNA. Data were analysed using SDS2.1 software (Applied Biosystems).

Microarray Analysis. Total cellular RNA was isolated using the miRNeasy mini kit (Qiagen), DNase treated (Ambion), quality analysed on an Agilent 2100 Bioanalyzer (Agilent Technologies) and further processed with the Ambion WT Expression Kit (Applied Biosystems) according to the manufacturers' instructions²⁹. cRNA was fragmented, labelled, and hybridised to the Affymetrix Human Gene 1.0 ST Arrays using the Gene Chip WT Terminal Labeling and Hybridization Kit (Affymetrix). GeneChip fluidics station 450 (Affymetrix) was used for processing of the arrays and fluorescent signals were detected with the GeneChip scanner 3000. Images were analysed with the GeneChip operating software (Affymetrix). Further analysis was performed with the Partek Genomics Suite (Partek). RMA processing and quantile normalization was applied, and after Median Polish and gene level probeset summarization, differentially expressed genes were identified using ANOVA. Data were submitted to Gene Expression Omnibus database (accession number GSE75603).

Western Blot Analysis. Total protein lysates were prepared using lysis buffer containing 1 mM protease inhibitor cocktail (Roche), 25 µg proteins loaded onto a 10% Bis-Tris NuPage gel (Invitrogen) and transferred onto a nitrocellulose membrane (Invitrogen). The membrane was incubated with primary antibodies against phospho-p44/42 MAPK and p44/p42 MAPK (Extracellular-signal-regulated kinase (ERK))(Cell Signaling) overnight at 4 °C, followed by secondary, horseradish peroxidase-conjugated antibody (goat anti rabbit IgG (Southern Biotech). Blots were developed using ECL plus Detection Reagent (GE Healthcare) and visualized on a Chemidoc MP System (BioRad). Data were analysed using Image Lab 4.1 software (BioRad).

ELISA. LAD2 and LUVA cells were stimulated for 6 hours in the presence of L-cysteine (3 mmol/L) with LTD₄ and LTE₄ (both 100 nmol/L) and vehicle control. CCL4 and CSF2 concentrations were measured in supernatants using human CCL4 (MIP-1β) and CSF2 (GM-CSF) duo set kits (R&D Systems, UK) following manufacturer's protocol.

Statistical analysis. Data were analysed by means of one- or two- way ANOVA using GraphPad Prism software (GraphPad). Differences were considered significant at a p-value of less than 0.05.

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Author Contributions

H.R.F., E.F., W.B. and G.W. performed the experiments and analysed data. G.W. and T.H.L. conceived and designed the experiments. D.J.C. contributed reagents/materials/analysis tools and analysed data. H.R.F., E.F., T.H.L. and G.W. wrote the paper. All authors reviewed and provided comments upon preparation of the manuscript.

Additional Information

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